Modulation of NR2B-regulated contextual fear in the hippocampus by the tissue plasminogen activator system

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Contextual fear conditioning is regulated by the hippocampus, and NR2B, a subunit of the NMDA receptor (NR), is involved in this process. We show that acute stress modulates tissue plasminogen activator (tPA) activity in the hippocampus by inducing expression of its inhibitor, plasminogen activator inhibitor-1. Acute stress increases NR2B expression and ERK1/2 phosphorylation, a classical marker of postsynaptic plasticity, in the hippocampus. tPA forms a complex with NR2B and is necessary for binding NR2B to postsynaptic density-95, allowing for NR activation and membrane anchoring. Acute stress increases the interaction between NR2B and RACK-1, which is also dependent on tPA, further suggesting that tPA is an important factor in NMDA signaling and plasticity in the hippocampus. Finally, acutely stressed tPA$^{-/-}$ mice show a decrease in contextual fear conditioning compared with stressed WT mice. These results indicate that tPA is a key modulator in stabilizing the NR complex during stress and participates in changes in behavior and synaptic plasticity.

fear conditioning | NMDA receptor | postsynaptic density-95 | plasminogen activator inhibitor

The hippocampus and amygdala are responsible for regulating the body's responses to stress (1, 2). Stressful situations damage the hippocampus by inducing atrophy and delaying neurogenesis, which inhibits memory formation and consolidation (3–8). Furthermore, repeated stress impairs hippocampal-dependent cognition and enhances amygdala-dependent unlearned fear and fear conditioning (7).

Hippocampal-based anxiety and learned fear can be studied by using contextual fear conditioning. Contextual fear depends on hippocampal formation and the amygdala (9, 10) and specifically involves NMDA receptor (NR) signaling (11–14). A disruption of contextual fear and deficits in long-term potentiation (LTP) are observed in mice with genetic or pharmacological alterations of NR subunits (15–20). This process also involves changes in synaptic plasticity and NR2B signaling in the hippocampus (14, 20–22), although the mechanism is unclear.

Tissue plasminogen activator (tPA) is a serine protease that is synthesized by and stored in neurons and is secreted during membrane depolarization (23–25). The activity of tPA is upregulated in the mouse amygdala after brief restraint stress (26). Amygdala-based behavioral experiments showed that stress prevented exploratory behaviors in WT mice but not in tPA$^{-/-}$ mice (26), indicating a critical role for tPA in regulating the amygdala stress response. However, the role of tPA during the stress response in the hippocampus has not been investigated.

tPA may act through a variety of protein–protein interactions as well as via proteolysis. Plasminogen activator inhibitor-1 (PAI-1) is a key inhibitor of tPA activity in the CNS, and a tPA/PAI-1 complex may act as a signaling molecule. The NR is an attractive target for tPA and/or the tPA/PAI-1 complex because tPA interacts with NR subunits (27–29). NRs and tPA each play roles in LTP, synaptic plasticity, and the stress response (24, 30). Here we show that tPA is a key regulator of contextual fear conditioning in the hippocampus via interactions with the NR.

Results

Acute Stress Induces Changes in the tPA System in the Hippocampus. To determine whether stress induces changes in the tPA system in the hippocampus, WT mice were acutely restrained and killed. Hippocampi were collected and prepared for biochemical and histochemical analyses. ELISAs were used to determine tPA protein levels, and in-gel and *in situ* zymographies were conducted to determine tPA activity. ELISAs showed that levels of total tPA protein remain unchanged after acute stress (data not shown). Although tPA protein expression is unaltered, tPA activity is greatly affected with acute stress. The most dramatic changes in tPA activity are shown in Fig. 1*A*. Overall, tPA activity was reduced by ≈55% after 6 hr of restraint (Fig. 1*B*). As a comparative study, *in situ* zymographies showed a decrease in tPA activity after 6 hr of restraint stress in the mossy fiber pathway of the hippocampus (Fig. 1*C*). To determine whether tPA activity was regulated by an inhibitor during the stress response in the hippocampus, PAI-1 expression was analyzed by ELISA (Fig. 1*D*). The level of total (free and complexed) PAI-1 was significantly increased in the hippocampus of WT mice after 6 hr of restraint, suggesting that PAI-1 was induced by stress in the hippocampus, leading to decreased tPA activity at this time point. PAI-1 levels in the hippocampi of stressed and nonstressed tPA$^{-/-}$ mice showed no significant difference, suggesting that changes in PAI-1 with stress are dependent on tPA. Furthermore, these results demonstrate that the decrease in tPA activity is attributable to inhibition rather than to a decline in tPA protein expression.

Stress-Induced Changes in Hippocampal Synaptic Plasticity Are Mediated by tPA. tPA is highly expressed in the hippocampus, the region responsible for learning and memory (31–33). Normally, tPA is up-regulated in hippocampal neurons shortly after induction of LTP (34), but tPA$^{-/-}$ mice show a decreased late phase of LTP (24, 35) and impaired learning in certain paradigms (35). In addition, overexpression or infusion of tPA into the hippocampus results in enhanced LTP and improved learning (36, 37). These results indicate a role for tPA in neuronal plasticity and memory formation.

Because of the correlation between tPA and LTP, we investigated the role of tPA in synaptic plasticity in the hippocampus during stress. WT and tPA$^{-/-}$ mice were restrained, and hippocampi were analyzed for changes in the levels of ERK1/2 and phosphorylated ERK1/2 (P-ERK1/2), a key marker of postsynaptic plasticity and learning (38, 39). Levels of ERK1/2 remained

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The authors declare no conflict of interest.

Abbrevations: LTP, long-term potentiation; NR, NMDA receptor; PAI-1, plasminogen activator inhibitor-1; P-ERK, phosphorylated ERK; PSD, postsynaptic density; tPA, tissue plasminogen activator.

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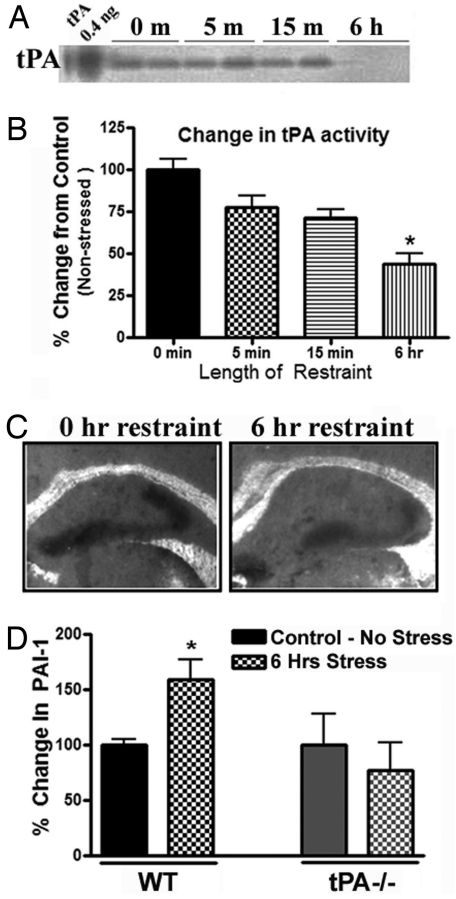

Fig. 1. Acute stress-induced changes in tPA and PAI-1 in the mouse hippocampus. Hippocampal homogenates from stressed and nonstressed WT mice were analyzed for tPA activity and PAI-1 expression. (*A*) In-gel zymography of hippocampal homogenates after 0 min, 5 min, 15 min, and 6 hr of restraint shows a dramatic change in tPA activity after 6 hr. (*B*) Quantification of in-gel zymographies from separate experiments ($n = 7–8$ per group). Amount of tPA activity after 6 hr of restraint stress is significantly reduced from earlier time points (*, $P < 0.0001$). (*C*) *In situ* zymographies from WT mouse hippocampi after 0 and 6 hr of restraint show a decrease in tPA activity in the mossy fiber pathway, comparable with results from in-gel zymographies. (*D*) PAI-1 ELISA shows a significant increase (*, $P = 0.0396$) in PAI-1 protein expression in hippocampal homogenates from WT mice restrained for 6 hr compared with those not restrained ($n = 4–6$ per group). However, no significant change in PAI-1 expression was found after restraint in tPA$^{-/-}$ mice.

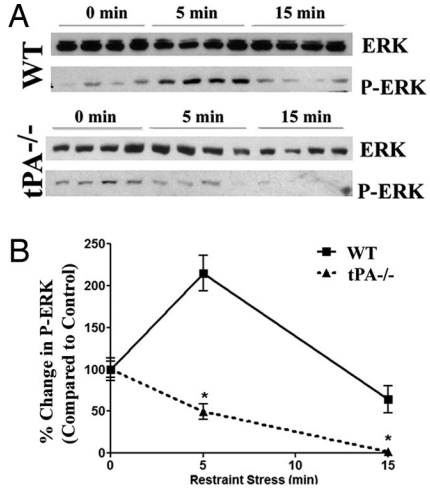

Fig. 2. Postsynaptic plasticity events are tPA-dependent in the hippocampus. (*A*) Western blot analysis of ERK1/2 and P-ERK in WT and tPA$^{-/-}$ hippocampal samples after 0, 5, or 15 min of restraint stress. (*B*) Quantification of ERK1/2 and P-ERK1/2 levels. Acute restraint stress causes a significant rise in P-ERK1/2 in the WT mouse hippocampus ($P = 0.0011$; WT, 0 vs. 5 min), which does not occur in tPA$^{-/-}$ mice (*, $P = 0.0005$; tPA$^{-/-}$ 5 min vs. WT 5 min). Levels of ERK1/2 remain constant throughout these time periods in both mouse lines. All samples were normalized to actin levels and then compared with WT control samples (0 min of stress) ($n = 4–7$ per group).

constant during this short-term restraint in WT and tPA$^{-/-}$ samples. P-ERK1/2 significantly increased after 5 min of restraint stress in WT mice but returned to normal by 15 min. However, P-ERK1/2 in tPA$^{-/-}$ mouse hippocampi decreased with brief restraint stress, which was significantly different in comparison with WT levels, and remained low (Fig. 2 *A* and *B*). These results implicate tPA as a critical player in synaptic plasticity events in the hippocampus during the response to stress. In the absence of tPA, cellular signaling and synaptic plasticity events may not properly take place, leading to abnormal phenotypes compared with controls.

Dependence of tPA on NR2B-Regulated Mechanisms During Acute Stress. To more fully examine the role of tPA in synaptic plasticity, cellular mechanisms known to be involved in hippocampal plasticity were analyzed during acute stress. tPA$^{-/-}$ mice have attenuated corticostriatal LTP, a form of synaptic plasticity that depends on the NR (40, 41), and are more sensitive to the disruption of LTP by NR antagonists during acquisition of a low-rate responding task (42). There is substantial evidence that tPA interacts with NR subunits. tPA causes an increase in intracellular calcium levels and promotes cell death in neuronal cultures treated with NMDA (27). Also, inhibition of tPA activity interferes with NMDA-dependent formation of perforated synapses in hippocampal cell culture (43). tPA regulates NR2B and its downstream signaling events during ethanol exposure and withdrawal in the hippocampus of WT mice (44). Therefore, we analyzed hippocampal homogenates from nonstressed and stressed WT and tPA$^{-/-}$ mice for NR2B and other molecules involved in its regulation. After acute stress, total NR2B levels significantly increased in WT mice but not in tPA$^{-/-}$ mice (Fig. 3*A*). The basal levels of NR2B in WT and tPA$^{-/-}$ mice were comparable by Western blot analysis (data not shown). These results further confirm tPA's regulation of NR2B expression in the mouse hippocampus, which may be at a transcriptional or translational level.

We previously showed an interaction between tPA and NR2B in the hippocampus of WT mice by coimmunoprecipitation (44). However, it was not known whether this interaction existed during the stress response. Hippocampal homogenates from stressed and nonstressed WT mice were immunoprecipitated to determine any direct interaction between NR2B and tPA during stress. Both stressed and nonstressed WT samples showed an interaction between tPA and NR2B, whereas tPA$^{-/-}$ samples (used as a negative control) did not (Fig. 3*B*). Therefore, the interaction between NR2B and tPA still exists during acute stress mechanisms in the hippocampus.

NR2B is a regulatory subunit of the NR (45) as its phosphorylation by Fyn kinase allows for cell-surface expression and receptor activity (46). Binding of postsynaptic density (PSD)-95 to NR2B anchors the receptor to the cell membrane and prevents its internalization (45). However, the scaffolding protein RACK-1 prevents Fyn-mediated phosphorylation of NR2B (47) and allows binding of adaptor protein-2 to promote endocytosis (46). Because there is a direct link between tPA and NR2B expression, we hypothesized that tPA alters NR2B–protein interactions during acute stress and consequently destabilizes the NR complex.

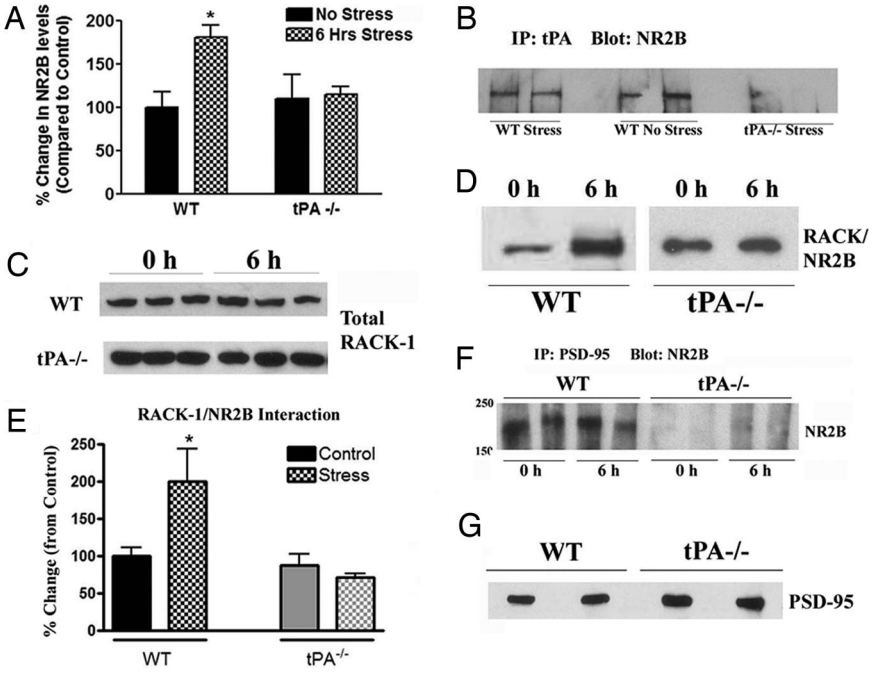

Fig. 3. tPA-dependent NR2B subunit changes during acute stress events in the hippocampus. (*A*) Total NR2B subunit levels significantly increase in WT mouse hippocampal homogenates after 6 hr of restraint stress (*, $P = 0.0047$; WT control vs. stress) but remain constant in tPA$^{-/-}$ mouse hippocampal homogenates after stress ($n = 7$–10 per group; all samples were normalized to actin before quantification). (*B*) Hippocampal homogenates from stressed WT and tPA$^{-/-}$ mice ($n = 4$ per group) were immunoprecipitated by using an anti-tPA antibody, and samples were subsequently Western-blotted and probed for NR2B. NR2B is immunoprecipitated from WT, but not tPA$^{-/-}$, mouse hippocampal homogenates, indicating an interaction between tPA and NR2B in the hippocampus. IP, immunoprecipitated. (*C* and *D*) Hippocampal homogenates from nonstressed and acutely stressed WT and tPA$^{-/-}$ mice were analyzed by Western blot for total levels of RACK-1 protein (*C*; normalized to actin) or immunoprecipitated with an anti-NR2B antibody (*D*). Immunoprecipitated extracts were subsequently Western blotted and probed for RACK-1. Samples from stressed WT mice show a significantly higher interaction between NR2B and RACK-1 than stressed tPA$^{-/-}$ samples, although levels of total RACK-1 remain constant with stress. (*E*) Bar graph shows quantification of changes in NR2B/RACK-1 interactions in nonstressed and stressed WT and tPA$^{-/-}$ mouse hippocampi ($n = 4$–8 per group). The interaction between NR2B and RACK-1 increases significantly in the WT mouse hippocampus after 6 hr of restraint stress (*, $P = 0.0291$; WT control vs. stress), whereas the interaction remains constant in the tPA$^{-/-}$ mouse hippocampus after stress. (*F*) Equal amounts of hippocampal homogenates from nonstressed and acutely stressed WT and tPA$^{-/-}$ mice ($n = 2$–3 per group) were immunoprecipitated (IP) with an anti-PSD-95 antibody. Subsequent Western blots were probed for NR2B. PSD-95 interacts with NR2B in the hippocampi of all WT mice but not in tPA$^{-/-}$ mice. (*G*) Hippocampal homogenates from nonstressed and acutely stressed WT and tPA$^{-/-}$ mice were analyzed by Western blot for total levels of PSD-95.

With immunoprecipitation of hippocampal homogenates with an anti-NR2B antibody, WT mice showed a significantly higher interaction between NR2B and RACK-1 than stressed tPA$^{-/-}$ samples (Fig. 3 *D* and *E*). These results were not attributable to changes in RACK-1 levels because Western blots indicated that total RACK-1 was unchanged in all samples, before and after stress (Fig. 3*C*). Similarly, homogenates were immunoprecipitated with an anti-PSD-95 antibody and probed for NR2B. Although there was no detectable difference in PSD-95/NR2B interactions in nonstressed and stressed WT mice, there was dramatically less of this interaction in nonstressed and stressed tPA$^{-/-}$ mice (Fig. 3*F*). Western blots of hippocampal lysates showed that the levels of PSD-95 were equivalent in both genotypes (Fig. 3*G*). These results indicate a pivotal role for tPA in PSD-95's interaction with NR2B; without tPA, the NR2B-containing NR complex is not stabilized as in normal conditions. These RACK-1 and PSD-95 results indicate a significant role for tPA in NR2B trafficking and NR complex stability in the hippocampus.

NR2B-Dependent Contextual Fear Is Modulated by tPA. Hippocampi of WT mice under acute stress conditions underwent changes in postsynaptic plasticity and NR2B-signaling events that were dependent on tPA (Figs. 2 and 3). It is well established that NRs are required for synaptic plasticity associated with learning and memory as well as contextual fear (14, 21, 48). Previous studies showed that stressed WT mice displayed exploratory deficits in the elevated plus maze, a task dependent on the amygdala, whereas tPA$^{-/-}$ mice did not show similar behaviors (26). To determine whether tPA has a hippocampal-based effect on behavior, contextual fear conditioning was used to analyze acutely stressed WT and tPA$^{-/-}$ mice.

WT and tPA$^{-/-}$ mice either remained unstressed or were restrained for 6 hr and allowed an 18-hr recovery period during the dark cycle. All animals displayed similar levels of fear (determined by percentage of freezing) to the context on the training day. Nonrestrained WT and tPA$^{-/-}$ mice responded with comparable levels of fear to the context on the testing day (data not shown), which is consistent with previously published work (35). However, WT and tPA$^{-/-}$ mice that were acutely restrained had a significantly different reaction to the context on the testing day (Fig. 4). Stressed WT mice froze ≈50% of the time during the testing session, whereas stressed tPA$^{-/-}$ mice only froze ≈25% of the time. Nonrestrained tPA$^{-/-}$ mice reacted similarly to WT mice, whereas restrained tPA$^{-/-}$ mice showed a lack of anxiety, suggesting that the deficiency in tPA prevents the molecular stress response, induced by restraint, from occurring in the hippocampus. Therefore, our fundamental finding is that stressed tPA$^{-/-}$ mice do not learn fear-conditioning tasks as well as stressed WT mice. These results implicate tPA as a critical regulator of the effects of short-term stress on learning and memory. Together, our results indicate an essential role for tPA in the hippocampal signaling processes involved in the stress response and implicate a behavioral role for tPA in the hippocampus.

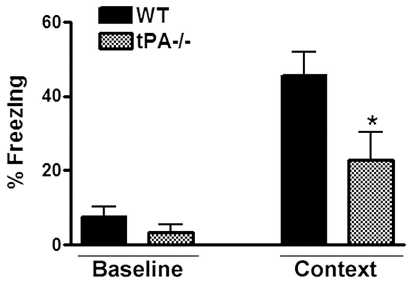

Fig. 4. tPA is necessary for contextual fear conditioning. WT ($n = 5$) and tPA$^{-/-}$ ($n = 7$) mice were restrained for 6 hr. After an 18-hr recovery period, mice were habituated and exposed to the fear-conditioning chamber. Baseline freezing levels were similar between WT and tPA$^{-/-}$ mice on the training day. Stressed tPA$^{-/-}$ mice displayed less fear than stressed WT mice during contextual (*, $P = 0.0402$) testing the following day.

Discussion

Acute restraint stress causes a slow decline of tPA activity in the hippocampus (Fig. 1 *A* and *B*), which is accompanied by an increase in PAI-1. Total tPA protein levels remain unchanged, indicating tPA is either active and unbound to PAI-1 or inactive and bound to PAI-1. The hippocampi of restrained tPA$^{-/-}$ mice do not contain heightened levels of PAI-1, indicating that this stress-induced increase depends on tPA.

The hippocampus and amygdala undergo neuronal plasticity in response to stress, and tPA plays an essential role in this plasticity in the amygdala (26). Molecules of the hippocampus, including tPA, are also involved in LTP, mechanisms of learning and memory, and stress responses (24, 34, 35, 41). We show here that the induction of P-ERK1/2 in the hippocampus, a key marker of postsynaptic activity and learning does not occur in stressed tPA$^{-/-}$ mice. Unlike the amygdala, presynaptic plasticity events in the hippocampus are not regulated by tPA, as levels of GAP43 remain consistent between WT and tPA$^{-/-}$ mice (data not shown). The changes in tPA activity, induced by stressful stimuli and the up-regulation of PAI-1, may induce changes in postsynaptic plasticity necessary for learning and remembering, hence leading to altered behaviors and phenotypes.

Altering the NR complex may modify synaptic plasticity and affect behavioral responses (11–13, 21, 48, 49). We further investigated the relationship between tPA and the NR complex and its downstream signaling mechanisms in WT and tPA$^{-/-}$ mice under nonstressed and stressed conditions. Levels of total NR2B are elevated in the hippocampus of WT, but not tPA$^{-/-}$, mice (Fig. 3*A*). Our results suggest that tPA interacts with NR2B under basal conditions and during stress (Fig. 3*B*). However, it is likely that the tPA that associates with NR2B is inactivated by the increased levels of PAI-1, whereas the tPA is active under basal conditions.

Stress stimulates an increase in NR2B/RACK-1 interactions in WT mice, but not in tPA$^{-/-}$ mice (Fig. 3 *D* and *E*). Furthermore, total levels of RACK-1 are unaltered in WT mice after restraint (Fig. 3*C*), whereas levels of NR2B are increased after acute stress in WT, but not tPA$^{-/-}$, mice (Fig. 3*A*). These results suggest that the stress-induced decrease in tPA activity initiates a signaling cascade that results in increased RACK-1 binding to NR2B. Again, it is likely that tPA is in an inactive form as a result of its interaction with PAI-1 during this signaling process because our results show a decrease in tPA activity and an up-regulation of PAI-1 after acute stress (Fig. 1). The increased NR2B/RACK-1 interaction suggests that the induction of PAI-1 and the reduction in tPA activity brought on by stress prevents phosphorylation of NR2B and hence prevents NR activation. Furthermore, our data showed that tPA is essential for PSD-95 binding to NR2B, although there is no detectable change in the NR2B/PSD-95 interaction after acute stress (Fig. 3*F*). These results indicate that although stress itself does not alter the PSD-95/NR2B interaction, the presence of tPA does affect it. The lack of NR2B/PSD-95 association in tPA$^{-/-}$ mice suggests that there may be altered NR subunit composition in the hippocampus of tPA$^{-/-}$ mice in comparison with that of WT mice. For example, hippocampal cells of tPA$^{-/-}$ mice may compensate by expressing more NR2A at the cell surface rather than NR2B. This hypothesis may also explain why tPA$^{-/-}$ mice display deficits in LTP (24, 35). Our results suggest the following molecular events in the hippocampus in response to acute stress in a normal situation (WT mouse): PAI-1 levels increase, leading to the down-regulation of tPA activity. The inactive tPA, which may act as a ligand, binds to NR2B and increases the association of RACK-1 with NR2B. The change in NR composition, localization, and its protein interactions may explain the alterations in synaptic plasticity as observed in stressed WT mice but not in tPA$^{-/-}$ mice.

To determine whether the hippocampal response to acute stress affects behavior, contextual fear conditioning was performed on WT and tPA$^{-/-}$ mice. Stressed WT mice froze in the presence of the learned context, whereas stressed tPA$^{-/-}$ mice froze significantly less. This behavioral deficit in tPA$^{-/-}$ mice may be caused by the lack of synaptic plasticity changes in the hippocampus, preventing these mice from learning and remembering to be fearful of the context during the training sessions. These tPA-dependent changes in synaptic plasticity may not have occurred in the tPA$^{-/-}$ mice because tPA was not present to interact with NR2B. This lack of the tPA/NR2B interaction then prevents PSD-95 stabilization of the NR complex, the increased interaction between NR2B and RACK-1, and the elevation in NR2B expression levels that are found in stressed WT mouse hippocampi. Fig. 5 shows a schematic of how acute stress, active and inactive forms of tPA, and PAI-1 may alter NR2B-containing NR protein interactions and consequently destabilize the receptor complex while also emphasizing the importance of tPA during synaptic plasticity and molecular events. Changes in the localization and activation of NR subunits may elicit some of the changes in synaptic plasticity, dendritic structure, and behaviors that are observed during the hippocampal stress response in mice.

One question that remains unanswered is the role of PAI-1 in the stress response. By using several biochemical criteria, PAI-1$^{-/-}$ mice have a similar response to stress as tPA$^{-/-}$ mice. Therefore, it will be important to explore the stress mechanism by using PAI-1$^{-/-}$ mice as a model and to better understand if and how a putative tPA/PAI-1 complex might interact with NR2B, PSD-95, and RACK-1 to induce changes in cell signaling, plasticity, and behavior. Moreover, the involvement of other tPA inhibitors is also a possibility. It will be interesting to determine whether neuroserpin, another common endogenous inhibitor of tPA, is involved in the stress pathway.

We have shown that tPA plays an essential role in the hippocampal response to stress and that PAI-1 may also be a key regulator in this pathway. These data provide an avenue for stress and anxiety research and may lead to more direct, targeted pharmacotherapies for patients with anxiety disorders.

Materials and Methods

Materials. Human recombinant tPA was provided by Genentech (South San Francisco, CA). Human plasma was provided by the New York Blood Center for the isolation of plasminogen for tPA zymographies. ELISA kits and recombinant PAI-1 were purchased from Molecular Innovations (Southfield, MI).

Antibodies. The following antibodies were used: anti-P-ERK1/2 and anti-ERK1/2 (Cell Signaling, Danvers, MA), anti-GAP43 (Chemicon International, Temecula, CA), anti-NR2B (PhosphoSolutions, Aurora, CO), anti-NMDAε2 C-20 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-RACK-1 (BD Transduc-

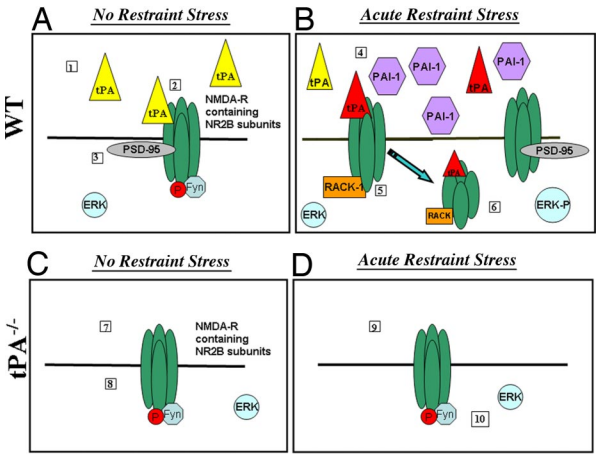

Fig. 5. Potential model for the role of tPA in NR2B-mediated mechanisms during synaptic plasticity and contextual fear conditioning. (*A*) Under normal conditions in the hippocampus, tPA is active (*1*) and interacts with NR2B subunits at the cell surface (*2*), and NR2B is anchored at the membrane by PSD-95 and is phosphorylated by Fyn kinase, allowing the NR to function normally (*3*). (*B*) After acute stress, PAI-1 levels increase, and PAI-1 inactivates tPA (*4*). This change in tPA activity leads to the inhibition of NR2B phosphorylation, which in turn promotes clathrin-mediated internalization (*5*). Total NR2B levels increase, as do interactions between NR2B and RACK-1 (*6*). The remaining active tPA (not inhibited by PAI-1) may interact with NR2B at the cell surface as in nonstressed conditions. Changes in cell-surface expression of NR subunits may hinder synaptic plasticity (via increased ERK1/2 phosphorylation, for example) and LTP, which may therefore lead to the anatomical and structural effects found in brains of stressed mice (3, 4, 6–8) as well as to the anxiety-like behaviors as demonstrated by fear conditioning and the elevated plus maze (26). (*C*) In tPA$^{-/-}$ mice under nonstressed conditions, the lack of tPA prevents its interaction with NR2B-containing NR in the hippocampus (*7*) and prevents NR2B/PSD-95 membrane stabilization (*8*). (*D*) After acute stress in the absence of tPA, NR2B levels are not up-regulated, PAI-1 expression is not induced, and NR2B is not stabilized at the membrane by PSD-95 (*9*); in addition, NR2B/RACK-1 interactions remain unchanged from control conditions, and ERK1/2 phosphorylation is not induced, all of which indicate a pivotal role for tPA in regulating these effects (*10*). In the absence of tPA, stress prevents any molecular changes and synaptic plasticity events from occurring in the hippocampus, which ultimately prevents stress-induced behaviors.

tion Laboratories, San Jose, CA), anti-PSD-95 (Synaptic Systems, Göttingen, Germany), anti-tPA (Molecular Innovations), and anti-β-actin (Sigma, St. Louis, MO).

Restraint Stress. Three-month-old WT C57BL/6 mice and tPA$^{-/-}$ mice backcrossed to C57BL/6 for nine generations were used. Mice were not disturbed for 2 weeks before experimentation to ensure that control animals were nonstressed. All experiments were conducted during the light cycle. Mice were stressed by using wire mesh restrainers secured at all sides. For biochemical experiments, mice were anesthetized with 2.5% avertin when released from restraints and perfused with PBS containing phosphatase inhibitors. Hippocampi were removed and frozen on dry ice. For behavioral experiments, mice were allowed 18 hr after release before testing. All procedures were approved by the Rockefeller University Institutional Animal Care and Use Committee, and all efforts were made to minimize suffering and the number of animals used.

tPA In-Gel Zymography. Hippocampi were dissected from nonstressed and stressed mice. Samples were homogenized in 100 mM Tris containing 0.2% Triton X-100, 0.2 mM sodium orthovanadate, and 10 mM sodium fluoride. After protein determination by the BCA assay (Pierce, Rockford, IL), hippocampal homogenates were adjusted to equal concentrations in nonreducing loading buffer and kept on ice. Samples were loaded on gels containing casein and plasminogen. Gels were then rinsed in 2.5% Triton X-100 twice for 30 min at 37°C and incubated overnight at 37°C in 0.25% Triton X-100. The gels were stained with Coomassie blue R-250 for 15 min and destained to visualize tPA activity. Activity was quantified with ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

***tPA in Situ* Zymography.** Mice were perfused with ice-cold PBS, and their brains were removed, frozen, and embedded in optimal cutting temperature compound. Zymographies were performed on coronal brain sections (20 μm) (26). To ensure proper comparisons, zymographies were developed, processed, and analyzed simultaneously.

Immunoprecipitation and Western Blotting. Samples of hippocampal homogenates adjusted to equal protein concentration were precleared with preimmune IgG and Gamma Bind Plus Sepharose beads (Amersham Biosciences, Piscataway, NJ). The antibody of interest was added for 1 hr, followed by incubation with Sepharose beads overnight at 4°C. Beads were washed with PBS and heated to 100°C for 5 min in loading buffer containing DTT. For Western blots, blots were blocked in 5% milk and incubated in primary antibody overnight at 4°C. Blots were rinsed in TBS containing 0.1% Tween 20 (TBS-T) and incubated in secondary antibody for 1 hr at room temperature followed by rinsing in TBS-T before exposure to film. Bands were scanned and quantified by ImageQuant software, and all samples were normalized to actin levels.

Contextual Fear Conditioning. Each conditioning chamber was equipped with a speaker, a house light, and a video camera. The chamber floors consisted of rods connected to a shock generator. Mice were restrained for 6 hr and allowed an 18-hr recovery period. Mice were habituated to the behavioral room for 1–2 hr on the day of training. During training, mice were placed into a conditioning chamber cleaned with an ammonia-based solution for 2 min, considered the "baseline period." Mice were then exposed to three tone–footshock pairings (tone, 20 sec, 85 dB, 3.5 kHz; footshock, 1 sec, 0.6 mA) with an intertrial interval of 60 sec. After 24 hr, mice were tested in the same chamber as the previous day for a 2-min period. All sessions were recorded on video to score the freezing behavior every 5 sec.

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